A new molecular identification tool for biting midges of the Obsoletus group (*Culicoides*, Ceratopogonidae): the 'glass slide' microarray approach

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Abstract

Culicoides species of the Obsoletus group (Diptera: Ceratopogonidae) are potential vectors of the bluetongue virus serotype 8 (BTV 8) that was introduced into central Western Europe in 2006. A major difficulty encountered is the correct morphological species identification of the Obsoletus group females, making molecular identification the method of choice. In this study we present a new molecular tool based upon probe hybridization using a DNA microarray format, to identify *Culicoides* species of the Obsoletus group. The Internal Transcribed Spacer 1 (ITS1) gene sequences of 55 *Culicoides* belonging to 13 different species were determined and used, together with 19 *Culicoides* ITS1 sequences from GenBank, to design species-specific probes for the microarray test. Evaluation of this test was performed using the amplified ITS1 sequences of another 85 *Culicoides* specimens, belonging to 11 species. The microarray test successfully identified all samples (100%) of the Obsoletus group, specifying each specimen to species level within the group. This test has several advantages over the existing PCR based molecular tools: the possibility for parallel analysis of many species, the high sensitivity and specificity, and low background signal noise. Hand-spotting of the microarray slide and the use of detection chemistry make this alternative technique affordable and feasible for any diagnostic laboratory with PCR facilities.

Keywords: bluetongue, molecular detection, *Culicoides obsoletus s.l.*, identification, Internal Transcribed Spacer 1, microarray, probe hybridization, vector

Introduction

Since the introduction of bluetongue virus serotype 8 (BTV 8) into central Western Europe in August 2006, many studies have been initiated to determine the species composition and distribution of biting midges of the genus *Culicoides* (Diptera: Ceratopogonidae) in the affected countries (Meiswinkel *et al.*, 2008). Initially the disease was limited to the Netherlands, Belgium and Germany but soon spread throughout Europe. At the end of 2008, holdings in 15 countries were affected by BTV 8 (Wilson & Mellor, 2009).

Several hypotheses were formulated about the introduction of the virus: import of viremic animals (domestic, wild or zoo-animals) or infected semen or embryos, or import of infected vectors (e.g., *Culicoides imicola* (Kieffer, 1913) of African origin, but presently well established in the Mediterranean area). These imports might have been the source of infection which was transmitted to local livestock making it possible for indigenous potential vectors to further transmit the virus (Carpenter *et al.*, 2009). Entomological surveys did not reveal the establishment of *C. imicola* or any other exotic vector species (Meiswinkel *et al.*, 2008), which implies that the disease has been spread by indigenous species.

Correct vector species identification is essential in the risk assessment of BTV transmission, as vectorial competence and capacity can vary significantly even between closely related species (Tabachnick, 2004). There are indications that *Culicoides* species of the Obsoletus and Pulicaris group, each consisting of morphologically and molecularly closely related species and even sibling species (e.g., *C. obsoletus* (Meigen, 1818) and *C. scoticus* (Downes & Kettle, 1952) belonging to the Obsoletus group), play a significant role in the spread of BTV (Mellor & Wittmann, 2002). Both species groups are abundant in BTV infected areas and virus has been

isolated from specimens belonging to these two groups during outbreaks in Italy (Caracappa *et al.*, 2003) and detected in these species using real-time PCR in central Western Europe (Hoffmann *et al.*, 2009; Vanbinst *et al.*, 2009; see also Carpenter *et al.*, 2008). Furthermore, in laboratory studies the Obsoletus group has been proven to be orally susceptible for BTV 8 and 9, and the Pulicaris group for BTV 9 (Carpenter *et al.*, 2006, 2008). As these species groups are morphologically hard to distinguish, taxonomic identification has been proven very difficult. Furthermore, the immature stages of the different *Culicoides* species are even harder to separate morphologically, which impedes ecological studies. In order to correctly identify potential vectors, a molecular based identification technique is essential.

Several molecular tools have been developed to identify *Culicoides* species or to study their phylogenetic relationship. Many focused on the Internal Transcribed Spacer 1 (ITS1) region of the ribosomal DNA (rDNA) to develop a rapid and reliable PCR (Cêtre-Sossah *et al.*, 2004), real time PCR (Cêtre-Sossah *et al.*, 2008) or multiplex PCR (Mathieu *et al.*, 2007) and to a lesser extent on the Internal Transcribed Spacer 2 (ITS2) rDNA (Gomulski *et al.*, 2006) and 18S rDNA (Kiehl *et al.*, 2009). Also PCR assays based on mitochondrial cytochrome oxidase subunit I (cox1) DNA have been used to study the phylogeny of the Imicola group (Linton *et al.*, 2002) and to identify species of the Obsoletus and Pulicaris groups (Pagès & Sarto I Monteys, 2005; Nolan *et al.*, 2007; Pagès *et al.*, 2009; Schwenkenbecher *et al.*, 2009; Augot *et al.*, 2010). However, a molecular identification technique based upon probe hybridization in a DNA microarray format (Schena *et al.*, 1995) has several advantages over these different PCR techniques: the possibility for parallel analysis of many species, the high sensitivity and specificity, and the low background signal noise (Zhou & Thompson, 2004). In this context, an easy and more economical alternative of the microarray technology has been recently developed

by De Witte (2007), which is applicable in any diagnostic laboratory with PCR facilities. In this study we present an adaptation of this alternative microarray test to identify *Culicoides* species of the Obsoletus group.

Materials and Methods

Sample collection and morphological identification

A total of 140 adult *Culicoides* specimens were selected from identified field samples collected with OVI 220V down-draught black light (8W) traps (Venter *et al*., 2009) in 2007 during the bluetongue vector monitoring program in Belgium (27 sampling locations) and the Grand Duchy of Luxembourg (1 sampling location). These were stored at the laboratory of animal health, Institute of Tropical Medicine Antwerp, in 80% ethanol and belonged to 13 different species. Fifty-five of these specimens were used for the development and 85 for the evaluation of the microarray test. Morphological identification of these specimens was based on a dichotomous key (Delécolle, 1985) and reference samples kindly offered by the taxonomic expert J.-C. Delécolle.

The nomenclature used in this paper is the one agreed on during the 2009 MEDREONET-meeting on taxonomy (Strasbourg, France, March 2009), and uses the following definitions: Species group: grouping of phylogenetically closely related species; morphological identification of most of the species in the group is possible (e.g.: Obsoletus group consists of females of the Obsoletus complex, *Culicoides montanus* (Shakirzjanova, 1962) (only recorded in Southern Europe), *Culicoides chiopterus* (Meigen, 1830), and *Culicoides dewulfi* (Goetghebuer, 1936)).

Species complex: grouping of phylogenetically closely related species, which are morphologically very hard/impossible to differentiate. These are also called sibling species (e.g.: Obsoletus complex consists of females of *C. obsoletus* and *C. scoticus*).

Sequencing and probe design

Nineteen ITS1 sequences from different *Culicoides* species retrieved from GenBank (Appendix A) were aligned. The specificity of the forward primer PanCulF (5'-GTA-GGT-GAA-CCT-GCG-GAA-GG-3') and the reverse primer PanCulR1 (5'-TGC-GGT-CTT-CAT-CGA-CCC-AT-3'), designed for the ITS1 sequence by Cêtre-Sossah *et al.* (2004), was confirmed by PCR. An extra reverse primer PanCulR2 (5'-TGC-GGT-CTT-CAT-CGA-TCC-AT-3') was designed to amplify the ITS1-region from Belgian *Culicoides* species. The use of two reverse primers was necessary because of a single nucleotide difference between the British and French sequences at the 3'region retrieved from GenBank. The mixture of those two reverse primers can also be noted as the degenerated reverse primer PanCulR1/R2 (5'-TGC-GGT-CTT-CAT-CGA-YCC-AT-3'). When performing PCR a mix of these two reverse primers was used to amplify all sequences. After PCR-reactions, using a proof reading Taq enzyme, 55 *Culicoides* samples belonging to 13 different, morphologically identified, species were cloned in *Escherichia coli* using TOPO TA Cloning® Kits (Invitrogen™) following the manufacturers' chemical transformation procedures. Positive clones were sent to VIB (Flemish Institute for Biotechnology, University of Antwerp) for sequencing. Retrieved sequences were aligned using BioEdit version 7.0.9 (Hall, 2007) and species-specific regions suited for probe design were selected.

All probes were designed in the reverse complement, and contained an amino linker at the 5'-end to bind onto the SAL-coated glass slides and a spacer region (TTT-TGT-TTT-GTT-TTG-TTT-T) to avoid steric hindrance.

Extraction

Extraction of DNA was achieved by incubating individual *Culicoides*, stored in 80% ethanol in Eppendorf tubes, at 95°C for 10 minutes in a 100 µl 10% Chelex®100 resin (Bio-Rad laboratories) suspension in TE buffer, using an Eppendorf Thermomixer Compact with constant shaking at 1200 RPM. After a brief centrifugation (30 seconds, 13000 RPM), the supernatant containing the DNA was transferred to a new tube and stored at 4°C till use.

DNA-amplification

An asymmetric PCR was performed by using the 5' Digoxigenin-labeled forward primer at a 10 times higher concentration than the reverse primers. The amplification reaction contained 50 mM KCl, 10 mM Tris, 1.65 mM $MgCl_2$, 10 µM labeled forward primer, 0.5 µM of each reverse primer, 0.8 mM dNTPs and 0.016 U/µl Taq polymerase. PCR was performed in a PTC-100 cycler (MJ research) using 55 cycles. The cycle employed was: 92°C for 30 seconds, 55°C for 45 seconds and 72°C for 60 seconds. Results were visualized on a 2% agarose gel, after 20 minutes electrophoresis at 100 V in 0.5x TAE buffer, using ethidium bromide staining.

Microarray preparation

On a commercial purchased SAL-coated glass slide (Asper Biotech Ltd) 2 µl of probe solution (100 µM oligo-concentration in a sodium carbonate/sodium bicarbonate solution) was spotted by hand, using a micropipette. To facilitate accurate positioning, a spot pattern on a paper template underneath the microscope slide was used. These spotted slides were then incubated at 37°C in a humid chamber for at least one hour. Afterwards the slides were submerged once in 1% ammonium hydroxide and twice in RO-DI water for 5 minutes and air dried before being used in the hybridization experiment.

Microarray hybridization

Of the labeled PCR product 10 µl was added to 99 µl of hybridization buffer (44% Milli-Q water, 30% formamide, 25% 20x SSPE, 1% SDS 10%) and 1 µl of 10 mg/ml salmon sperm DNA (Invitrogen). The solution was dispersed onto the spotted area of the slide and incubated at 37°C in a covered humid chamber for at least 16 hours. Afterwards slides were washed 3 times for 5 minutes in Cobas Amplicor™ wash buffer (Roche), followed by a 5 minutes wash in Phosphate Buffered Saline (PBS).

Visualization of the hybridized Digoxigenin labeled DNA fragment was preceded by blocking of the slide by incubation with 2ml PBS containing 1% newborn calf serum (NBCS) for 30 minutes at room temperature. This was followed by incubation with anti-Digoxigenin monoclonal antibodies (Sigma-Aldrich) diluted 1/1000 in 2 ml PBS containing 1% NBCS, at room temperature for 1-2 hours. Consecutively the slides were washed twice for 5 minutes in PBS containing 0.05% Tween 20% and once for 5 minutes in PBS. After this, visualization was

achieved using Vector Blue alkaline phosphatase substrate kit III (Vector Laboratories) according to the manufacturers' protocol.

Results

The most abundant, morphologically identified *Culicoides* species observed at the sampling sites belonged to the Obsoletus group i.e., *C. obsoletus, C. dewulfi, C. chiopterus* and *C. scoticus*. Other frequently encountered species were *Culicoides pulicaris* (Linnaeus, 1758)*, Culicoides punctatus* (Meigen, 1804)*, Culicoides achrayi* (Kettle & Lawson, 1955), *Culicoides festivipennis* (Kieffer, 1914) and *C. nubeculosus* (Meigen, 1830).

Probe design: alignment of the ITS1 rDNA of Culicoides

Fifty-five morphologically identified Belgian *Culicoides* specimens (see Appendix B for details) belonging to 13 species (Obsoletus group (23): *C. obsoletus* (4), *C. scoticus* (7) *C. dewulfi* (6), *C. chiopterus* (6); Pulicaris group (26): *C. pulicaris* (6), *C. punctatus* (4), *Culicoides lupicaris* (Downes & Kettle, 1952) (5), *Culicoides newsteadi* (Austen, 1921) (5), *Culicoides impunctatus* (Goetghebuer, 1920) (4), *Culicoides deltus* (Edwards, 1939) (2); Nubeculosus group (6): *C. nubeculosus* (4), *Culicoides riethi* (Kieffer, 1914) (1), *Culicoides puncticollis* (Becker,1903) (1)) were sequenced. The sequences of the four Obsoletus group species were submitted to GenBank (Appendix C). After alignment with the British and French ITS1 sequences, a common *Culicoides* probe was designed in a highly conserved region (from 186 to 208 bp): 5'-GCT-CTT-CTA-TAA-AGC-TAC-CCA-AG-3' (Fig. 1) and specificity was tested using a Basic Local Alignment Search Tool for nucleotides (BLASTn) against known ITS1 sequences of *Culicoides*

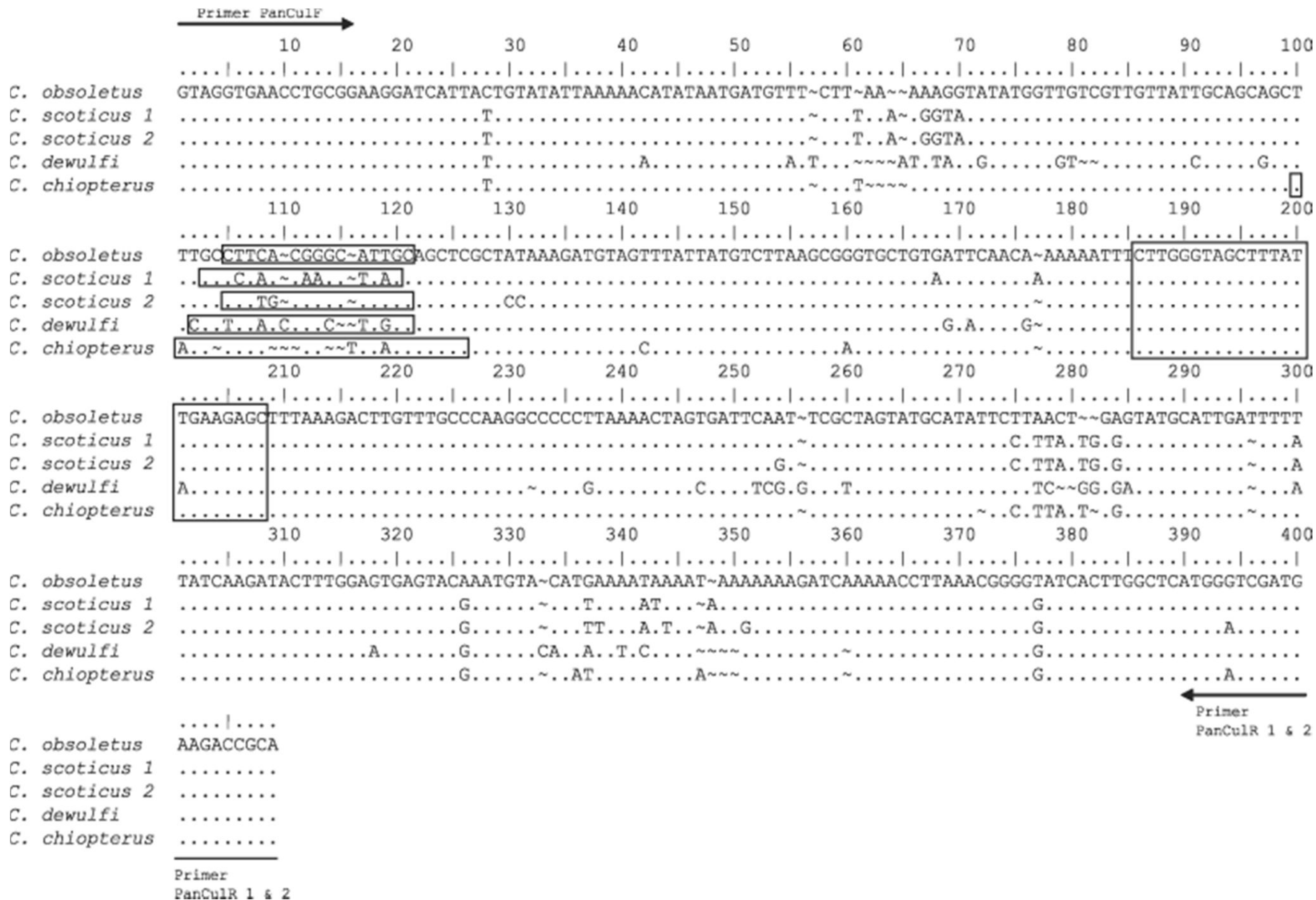

Fig. 1. Comparison of the ITS1 sequences from the four species of the Obsoletus group and indication of the species-specific and common probe regions.

(Appendix A), Culicidae, Psychodidae and Simuliidae species (Appendix D). This resulted in unique hits with species of the genus *Culicoides*.

Species-specific regions were located in a common region (from approximately 100 to 126 bp) (Fig. 1). In this region six species-specific probes were designed for the four species from the Obsoletus group, taking into account base pair-length and GC content (Table 1). Sequence alignment revealed two haplotypes in *C. scoticus*, each necessitating a different probe. After testing different extracts and cloned ITS1 products of *C. scoticus* with the microarray assay (unpublished data), it was clear that two different alleles of the ITS1 region of *C. scoticus* exist. For *C. chiopterus* an alternative probe was designed, as the original probe often gave false-negative binding. But after optimization of the protocol (mainly regarding temperature) both probes were found to be specific only for *C. chiopterus* and either one can be used in the test. The specificity of probe targets for the respective species was tested using a BLASTn against the database of all 65 currently known *Culicoides* species ITS1 sequences (Appendix A). This resulted in unique hits with the respective species probes.

Molecular identification of four species from the Obsoletus group by the microarray test

During optimization and validation of the microarray test, 85 *Culicoides* specimens were morphologically identified by experts. These specimens, belonging to 11 *Culicoides* species (Obsoletus group (25): *C. obsoletus* (4), *C. scoticus* (8), *C. dewulfi* (8), *C. chiopterus* (5); Pulicaris group (57): *C. pulicaris* (11), *C. punctatus* (6), *C. lupicaris* (10), *C. pulicaris/lupicaris* intermediate (11), *C. newsteadi* (6), *C. impunctatus* (2), *C. deltus* (11) and *C. nubeculosus* (3)),

Table 1

Nucleotide sequence of the designed Obsoletus group probes and their characteristics (Tm = melting temperature, calculated with OligoCalc version 3.26 (Kibbe, 2007)).

Species	**Sequence (5'-3')**	**Tm (ºC)**	**GC content (%)**
C. obsoletus	GCA-ATG-CCC-GTG-AAG	45	60
C. scoticus 1	CTA-AGC-TTG-TTA-GGG-C	43	50
C. scoticus 2	GCA-ATG-CCC-GCA-AAG	45	60
C. dewulfi	GCC-AAG-CCG-GTT-AAA-G	46	56
C. chiopterus	GAG-CTG-CTA-TAC-CGA-AG	47	53
C. chiopterus alternative	GAG-CTG-CTA-TAC-CGA-AGC-ATA	54	52

were assayed in the microarray test according to the described methodology (Fig. 2a). All PCR products from species samples of the Obsoletus group showed positive hybridization with the species-specific and common probes (Fig. 2b). Positive results were characterized by a blue colored spot at the specific probe site. As expected, other *Culicoides* species only hybridized with the common probe.

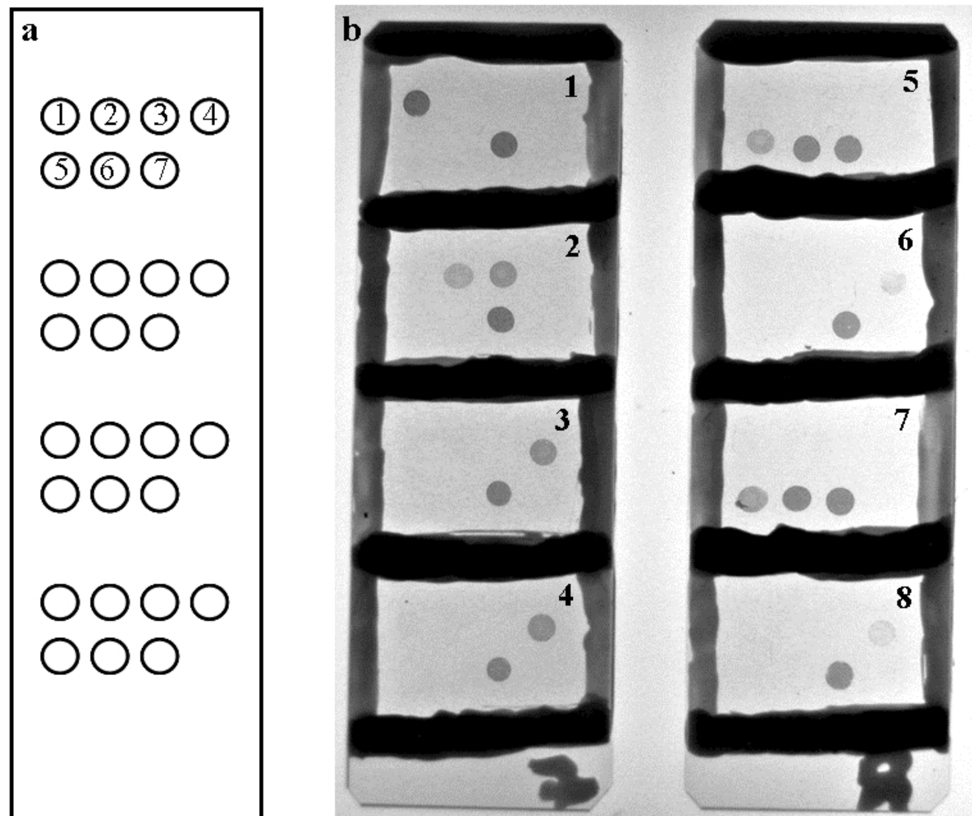

Fig. 2. a) Spotting of the seven probes on the SAL-coated glass slide (1: *Culicoides obsoletus*, 2: *C. scoticus* 1, 3: *C. scoticus* 2, 4: *C. dewulfi*, 5: *C. chiopterus*, 6: *C. chiopterus* alternative & 7: common *Culicoides* probe). b) Microarray hybridization of the Obsoletus group species with their species-specific and the common probe (1: *C. obsoletus*, 2: *C. scoticus*, 3: *C. dewulfi*, 4: *C. dewulfi*, 5: *C. chiopterus*, 6: *C. dewulfi*, 7: *C. chiopterus* & 8: *C. dewulfi*).

Discussion

Species belonging to the Obsoletus group are potential bluetongue vectors (Mellor & Pitzolis, 1979; De Liberato, 2005) but, since females of the Obsoletus group (especially of the Obsoletus

complex) are difficult to identify to species level, exact vector species of BTV could not be identified in the UK and Belgium (Carpenter *et al.*, 2006; Vanbinst *et al.*, 2009).

We have shown in this study that the microarray concept is useful for the correct identification of *Culicoides* species belonging to the Obsoletus group. Furthermore, this was also confirmed by the results of three EU-MEDREONET ring trials on molecular identification of species from the Obsoletus group (*C. obsoletus*, *C. scoticus*, *C. dewulfi* and *C. chiopterus*), in which the microarray technique correctly identified all 72 specimens (Cêtre-Sossah *et al.*, 2010).

In our initial experiments, we established the optimal hybridization conditions of the microarray test in order to reduce the number of false-positive and false-negative hybridization events. Good hybridization with the species-specific probes in combination with absence of signals on the negative probes was only detected at 37ºC. Absence of hybridization or faint signals to some probes at higher temperatures (40ºC to 44ºC) was probably due to low hydrostatic forces among complementary bases. Hybridization at room temperature was not tried as it was reported in Peplies *et al.* (2003) that secondary structures of the target molecule become more stable at low temperatures. This might result in reduced accessibility of the probe binding sites increasing the likelihood of false-negative results.

Considering possible interferences due to competition between the complementary strand of the double stranded PCR product and the probe (Guo *et al.*, 1994), only single stranded PCR products from an asymmetric PCR were used in this study. As positive results were obtained, we did not investigate the true influence of complementary double stranded PCR products on binding efficiency to the probes. Ideally the optimal GC content of a probe should be around 40% and melting temperatures should all be in the same range since all reactions are performed

simultaneously on the same slide. Due to few variable regions in the ITS1 sequence, this could not be attained. Therefore most of the probes did not have the theoretical optimal properties but no adverse effects were observed. In order to reduce the negative effects of steric hindrance on hybridization, a spacer of 15 extra base pairs between glass support and the actual probe was added.

Up till now, morphological identification keys and several PCR techniques are used for the identification of *Culicoides* (e.g., Campbell & Pelham-Clinton, 1960; Delécolle, 1985; Mathieu *et al.*, 2007). However, in many cases, so called intermediate adult morphs (e.g., in the Pulicaris group) will hamper correct morphological identification or even make this impossible. In contrast to PCR-assays, a microarray test will specifically detect inter- and intra-species differences as different probes can be developed for one or more species-specific regions (e.g., the heterozygote alleles of *C. scoticus*). In addition, species-specific detection is based upon visualization of positive hybridizations through different patterns of blue-colored spots corresponding to the species (or ‘intermediate’ species) and not on the length of the PCR product. The latter was found to be problematic when using multiplex PCR (based on Mathieu *et al.*, 2007) because of the formation of non-specific bands, primer dimers or weak signals caused by inhibiting interactions between primers (unpublished data). Moreover, simple PCR, multiplex PCR and real time PCR can only identify for a small number of species simultaneously.

A possible shortcoming of this method, as for molecular tools in general, is that the original specimen is destroyed in the identification process. As the microarray can be performed on parts of the specimens (legs) without destroying the original specimen as a whole, "microarray negative specimens" can be re-evaluated morphologically. Moreover, the inclusion of a common

Culicoides probe in the assay will signal yet undescribed species and further sequence analysis can be undertaken.

This study shows that a DNA microarray test based on the ITS1 region could serve as a reliable alternative for the identification of *Culicoides* species. Table 2 compares the cost/specimen, reaction time and specificity of the microarray test with those of the most commonly used identification techniques for *Culicoides* species of the Obsoletus group. The specificity of the microarray test is 100%, also for *C. obsoletus* and *C. scoticus* of the Obsoletus complex, in contrast to multiplex PCR (based on Mathieu *et al.*, 2007) and morphological identification. An unambiguous identification of these two species through the microarray test will not only improve the baseline data of their circulation in a certain area, but will also be important to investigate their behavior as well as biology including vector competence. The latter can be achieved by combining both the microarray with virus detection or isolation, which is currently under investigation.

Finally, the development of probes for more *Culicoides* species that are relatively common in Belgium and Western Europe is also ongoing. Theoretical probe sequences were selected and actual probes are currently under development for the following species: *C. nubeculosus*, *C. puncticollis*, *C. achrayi*, *Culicoides stigma* (Meigen, 1818), *Culicoides parroti* (Kieffer, 1922), *Culicoides pictipennis* (Staeger, 1839) and *Culicoides kibunensis* (Tokunaga, 1937). Probes for the species of the Pulicaris group are also being designed and tested.

In conclusion, a new and simple DNA microarray assay for identification of *Culicoides* belonging to the Obsoletus group in Western Europe has been developed and evaluated. The

Table 2

Comparison of the cost/specimen, reaction time and specificity of the microarray test with those of the most commonly used identification techniques for the Obsoletus group species: morphological identification and multiplex PCR.

	Microarray	**Multiplex PCR**	**Morphology**
Cost/specimen (€)	12.75	12	< 1, but expertise essential
Reaction time	2 days	1 day	< 3 min for experts
Specificity	100%	86%[a]	81%[a], 83%[b]

[a]only tested for females of the Obsoletus complex (multiplex PCR: n = 183, morphological identification: n = 158) (unpublished data)

[b]Pagès & Sarto I Monteys (2005)

assay was designed in such a manner that the need for expensive laboratory equipment and reagents was kept as low as possible making the technique affordable and feasible for any diagnostic laboratory with PCR facilities. Especially the detection chemistry in this method allows interpretation of the results by the naked eye thereby eliminating the need for the expensive laser-scanning devices often used in microarray experiments. Therefore the same approach can be applied for differentiating well defined and stable polymorphisms including any kind of identification analysis based on hybridization.

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References

Augot, D., Sauvage, F., Jouet, D., Simphal, E., Veuille, M., Couloux, A., Kaltenbach, M.L. & Depaquit J. (2010) Discrimination of *Culicoides obsoletus* and *Culicoides scoticus*, potential bluetongue vectors, by morphometrical and mitochondrial cytochrome oxidase subunit I analysis. *Infection, Genetics and Evolution*, **10**, 629–637.

Campbell, J.A. & Pelham-Clinton, E.C. (1960) A taxonomic review of the British species of *Culicoides* Latreille (Diptera: Ceratopogonidae). *Proceedings of the Royal Society of Edinburgh (B)*, **67**, 181-302.

Caracappa, S., Torina, A., Guercio, A., Vitale, F., Calabro, A., Purpari, G., Ferrantelli, V., Vitale, M. & Mellor, P.S. (2003) Identification of a novel bluetongue virus vector species of *Culicoides* in Sicily. *Veterinary Record*, **153**, 71-74.

Carpenter, S., Lunt, H.L., Arav, D., Venter, G.J. & Mellor, P.S. (2006) Oral susceptibility to bluetongue virus of *Culicoides* (Diptera: Ceratopogonidae) from the United Kingdom. *Journal of Medical Entomology*, **43**, 73-78.

Carpenter, S., McArthur, C., Selby, R., Ward, R., Nolan, D.V., Mordue Luntz, A.J., Dallas, J.F., Tripet, F. & Mellor, P.S. (2008) Experimental infection studies of uk *Culicoides* species midges with bluetongue virus serotypes 8 and 9. *Veterinary Record*, **163**, 589-592.

Carpenter, S., Wilson, A. & Mellor, P.S. (2009) *Culicoides* and the emergence of bluetongue virus in northern Europe. *Trends in Microbiology*, **17**, 172-178.

Cêtre-Sossah, C., Baldet, T., Delecolle, J.C., Mathieu, B., Perrin, A., Grillet, C. & Albina, E. (2004) Molecular detection of *Culicoides* spp. and *Culicoides imicola*, the principal vector of bluetongue (BT) and African horse sickness (AHS) in Africa and Europe. *Veterinary Research*, **35**, 325-337.

Cêtre-Sossah, C., Mathieu, B., Setier-Rio, M.-L., Grillet, C., Baldet, T., Delécolle, J.-C. & Albina, E. (2008) Development and evaluation of a real-time quantitative PCR assay for *Culicoides imicola*, one of the main vectors of bluetongue (BT) and African horse sickness (AHS) in Africa and Europe. *Research in Veterinary Science*, **85**, 372-382.

Cêtre-Sossah, C., Balenghien, T., Martinez, D., Albina, E., Garros, C., Giovannoni, V., Conte, A., Miranda, M.A., Baylis, M., Mertens, P., Carpenter, S., Domingo, M., Casal, J., Zientara, S., Stärk, K., Schwermer, H., Madani, H., Hammami, S., Sghaier, S., Lhor, Y., Ertürk, A., Georgiev, G., Sanchez-Vizcaino, J.M., Patakakis, M., Boinas, F., Venter, G., Beer, M., Hoffmann, B., De Clercq, K., Van Rijn, P.C.J. & Botner, A. (2010) Strengthening cooperation on vectorborne diseases in Europe. *Veterinary Record*, **166**, 386-387.

Delécolle, J.C. (1985) Nouvelle contribution à l'étude systématique et iconographique des espèces du genre *Culicoides* (Diptera: Ceratopogonidae) du Nord-Est de la France. PhD Dissertation. Université Louis Pasteur de Strasbourg, UFR des Sciences de la Vie et de la Terre, Strasbourg.

De Liberato, C., Scavia, G., Lorenzetti, R., Scaramozzino, P., Amaddeo, D., Cardeti, G., Scicluna, M., Ferrari, G. & Autorino, G.L. (2005) Identification of *Culicoides obsoletus* (Diptera: Ceratopogonidae) as a vector of bluetongue virus in central Italy. *Veterinary Record*, **156**, 301-304.

De Witte, J.C. (2007) DNA sequence specific method for analysis of *Theileria parva* diversity using microarrays. MSc Thesis. Intstitute of Tropical Medicine, Antwerp.

Gomulski, L.M., Meiswinkel, R., Delécolle, J.-C., Goffredo, M. & Gasperi, G. (2006) Phylogeny of the subgenus *Culicoides* and related species in Italy, inferred from internal transcribed spacer 2 ribosomal DNA sequences. *Medical and Veterinary Entomology*, **20**, 229-238.

Guo, Z., Guilfoyle, R.A., Thiel, A.J., Wang, R. & Smith, L.M. (1994) Direct fluorescence analysis of genetic polymorphisms by hybridization with oligonucleotide arrays on glass supports. *Nucleic Acids Research*, **22**, 5456-5465.

Hall, T. (2007) BioEdit v7.0.9. www.mbio.ncsu.edu/BioEdit/bioedit.html. [accessed on 16 January 2009]

Hoffmann, B., Bauer, B., Bauer, C., Bätza, H.-J., Beer, M., Clausen, P.-H., Geier, M., Gethmann, J.M., Kiel, E., Liebisch, G., Liebisch, A., Mehlhorn, H., Schaub, G.A., Werner, D. & Conraths,

F.J. (2009) Monitoring of putative vectors of bluetongue virus serotype 8, Germany. *Emerging Infectious Diseases*, **15**, 1481-1484.

Kibbe, W.A. (2007) OligoCalc: an online oligonucleotide properties calculator. *Nucleic Acids Research*, **35**, W43-W46.

Kiehl, E., Walldorf, V., Klimpel, S., Al Quraishy, S. & Mehlhorn, H. (2009) The European vectors of Bluetongue virus: are there species complexes, single species or races in *Culicoides obsoletus* and *C. pulicaris* detectable by sequencing ITS-1, ITS-2 and 18S-rDNA? *Parasitology Research*, **105**, 331-336.

Linton, Y.-M., Mordue (Luntz), A.J., Cruickshank, R.H., Meiswinkel, R., Mellor, P.S. & Dallas, J.F. (2002) Phylogenetic analysis of the mitochondrial cytochrome oxidase subunit I gene of five species of the *Culicoides imicola* species complex. *Medical and Veterinary Entomology*, **16**,139-146.

Mathieu, B., Perrin, A., Baldet, T., Delécolle, J.-C., Albina, E. & Cêtre-Sossah, C. (2007) Molecular identification of Western European species of *obsoletus* complex (Diptera: Ceratopogonidae) by an internal transcribed spacer-1 rDNA multiplex polymerase chain reaction assay. *Journal of Medical Entomology*, **44**, 1019-1025.

Meiswinkel, R., Baldet, T., De Deken, R., Takken, W., Delécolle, J.-C. & Mellor, P.S. (2008) The 2006 outbreak of bluetongue in northern Europe – the entomological perspective. *Preventive Veterinary Medicine*, **87**, 55-63.

Mellor, P.S. & Pitzolis, G. (1979) Observations on breeding sites and light-trap collections of *Culicoides* during an outbreak of bluetongue in Cyprus. *Bulletin of Entomological Research*, **69**, 229-234.

Mellor, P.S. & Wittmann, E.J. (2002) Bluetongue virus in the Mediterranean Basin 1998-2001. *The Veterinary Journal*, **164**, 20-37.

Nolan, D.V., Carpenter, S., Barber, J., Mellor, P.S., Dallas, J.F., Mordue (Luntz), A.J. & Piertney S.B. (2007) Rapid diagnostic PCR assays for members of the *Culicoides obsoletus* and *Culicoides pulicaris* species complexes, implicated vectors of bluetongue virus in Europe. *Veterinary Microbiology*, **124**, 82-94.

Pagès, N. & Sarto I Monteys, V. (2005) Differentiation of *Culicoides obsoletus* and *Culicoides scoticus* (Diptera: Ceratopogonidae) based on mitochondrial cytochrome oxidase subunit I. *Journal of Medical Entomology*, **42**, 1026-1034

Pagès, N., Munoz-Munoz, F., Talavera, S., Sarto, V., Lorca, C. & Nunez, J.I. (2009) Identification of cryptic species of *Culicoides* (Diptera: Ceratopogonidae) in the subgenus *Culicoides* and development of species-specific PCR assays based on barcode regions. *Veterinary Parasitology*, **165**, 298-310.

Peplies, J., Glockner, F.O. & Amann, R. (2003) Optimization strategies for DNA microarray-based detection of bacteria with 16S rRNA-targeting oligonucleotide probes. *Applied and Environmental Microbiology*, **69**, 1397-1407.

Schena, M., Shalon, D., Davis, R.W. & Brown, P.O. (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science*, **270**, 467 – 470.

Schwenkenbecher, J.M., Mordue (Luntz), A.J., Switek, K. & Piertney, S.B. (2009) Discrimination of *Culicoides* midge larvae using multiplex polymerase chain reaction assays based on DNA sequence variation at the mitochondrial cytochrome C oxidase I gene. *Journal of Medical Entomology*, **46**, 610-614.

Tabachnick, W.J. (2004) *Culicoides* and the global epidemiology of bluetongue virus infection. *Veterinaria Italiana*, **40**, 145-150.

Vanbinst, T., Vandenbussche, F., Vandemeulebroucke, E., De Leeuw, I., Deblauwe, I., De Deken, G., Madder, M., Haubruge, E., Losson, B. & De Clercq, K. (2009) Bluetongue virus detection by real-time RT-PCR in *Culicoides* captured during the 2006 epizootic in Belgium and development of an internal control export. *Transboundary and Emerging Diseases*, **56**, 170-177.

Venter, G.J., Labuschagne, K., Hermanides, K.G., Boikanyo, S.N.B., Majatladi, D.M. & Morey, L. (2009) Comparison of the efficiency of five suction light traps under field conditions in South Africa for the collection of *Culicoides* species. *Veterinary Parasitology*, **166**, 299-307.

Wilson, A. J. & Mellor, P. S. (2009) Bluetongue in Europe: past, present and future. *Philosophical Transactions of the Royal Society B*, **364**, 2669-2681.

Zhou, J. & Thompson, D.K. (2004) Microarray technology and applications in environmental microbiology. *Advances in Agronomy, vol. 82*. (ed. by D. L. Sparks), pp. 183-270. Elsevier Inc., San Diego, CA.

Appendix A. GenBank accession numbers of 65 *Culicoides* ITS1 sequences used for the sequence alignment (*) and local BLASTn searches.

Species	**GenBank accession number**	**Country**
C. actoni	AB462259	Japan
C. albicans	AJ417980*	United Kingdom
C. arakawae	AB462265	Japan
C. arakawae	AJ489503	China
C. aterinervis	AB462267	Japan
C. brevipalpis	AB462260	Japan
C. brevitarsis	AB462261	Japan
C. chiopterus	DQ408543*	France
C. circumscriptus	AY861163*	France
C. cataneii	AY861139	France
C. charadraeus	AB462277	Japan
C. cubitalis (=*kibunensis*)	AJ417979*	United Kingdom
C. cylindratus	AB462268	Japan
C. cylindratus	AB462269	Japan

C. derisor	AY861140	France
C. dewulfi	DQ408545*	France
C. dubius	AB462270	Japan
C. erairai	AB462278	Japan
C. festivipennis	AY861141*	France
C. griseidorsum	AY861142	France
C. grisescens	AJ417987*	United Kingdom
C. heteroclitus	AY861143	France
C. humeralis	AB462282	Japan
C. imicola	AY861144	France
C. imicola	AF074019	Israël
C. impunctatus	AJ417986*	United Kingdom
C. impunctatus	AJ417985*	United Kingdom
C. indistinctus	AY861145	France
C. jacobsoni	AB462262	Japan
C. japonicus	AB462266	Japan
C. kibunensis	AY861146	France

C. kibunensis	AB462280	Japan
C. kurensis	AY861147	France
C. lungchiensis	AB462271	Japan
C. lupicaris	AY861148*	France
C. maculatus	AB462263	Japan
C. malevillei	AY861149	France
C. maritimus	AY861150	France
C. maritimus	AJ417981	United Kingdom
C. matsuzawai	AB462283	Japan
C. montanus	DQ408544*	France
C. newsteadi	AY861151*	France
C. nipponensis	AB462272	Japan
C. nubeculosus	AJ417982*	United Kingdom
C. obsoletus	AY861152*	France
C. ohmorii	AB462273	Japan
C. oxystoma	AB462279	Japan
C. paraflavescens	AB462284	Japan

C. parroti	AY861153*	France
C. peregrinus	AB462274	Japan
C. pictipennis	AY861154	France
C. picturatus	AY861155	France
C. pulicaris	AY861156*	France
C. pulicaris	AJ417983*	United Kingdom
C. punctatus	AY861157*	France
C. punctatus	AB462275	Japan
C. puncticollis	AY861158	France
C. sahariensis	AY861159	France
C. scoticus	AY861160*	France
C. subfagineus	AY861161	France
C. submaritimus	AY861162	France
C. sumatrae	AB462276	Japan
C. variipennis	U48380	USA
C. verbosus	AB462281	Japan
C. wadai	AB462264	Japan

Appendix B. List of all 55 *Culicoides* sequenced, their laboratory ID numbers and location where they were caught. (* species caught in the Grand Duchy of Luxembourg, no geographical coordinates)

Species	ID	Location	Geographical coordinates
C. chiopterus	BT225	Betekom	N°: 51.00490; E°: 4.79240
	BT226	Sint Laureins	N°: 51.27365; E°: 3.55620
	BT383	Varendonk	N°: 51.085820; E°: 4.954160
	BT384	Ekeren	N°: 51.28333; E°: 4.41667
	BT385	Knokke	N°: 51.340250; E°: 3.352130
	ITM_CH	Belgium	Not defined
C. deltus	BT238	Gembloux	N°: 50.56263; E°: 4.70960
	BT239	Gembloux	N°: 50.56263; E°: 4.70960
C. dewulfi	BT219	Betekom	N°: 51.00490; E°: 4.79240
	BT220	Sint Laureins	N°: 51.27365; E°: 3.55620
	BT380	Manage	N°: 50.485950; E°: 4.215720
	BT381	Varendonk	N°: 51.085820; E°: 4.954160
	BT382	Betekom	N°: 51.00490; E°: 4.79240
	ITM_DE	Belgium	Not defined
C. impunctatus	BT240	Betekom	N°: 51.00490; E°: 4.79240
	BT241	Betekom	N°: 51.00490; E°: 4.79240
	BT401	Betekom	N°: 51.00490; E°: 4.79240
	BT400	Varendonk	N°: 51.085820; E°: 4.954160
C. lupicaris	BT234	Gembloux	N°: 50.56263; E°: 4.70960
	BT235	Gembloux	N°: 50.56263; E°: 4.70960
	BT392	Christnach*	
	BT393	Christnach*	

	BT394	Christnach*	
C. newsteadi	BT236	Varendonk	N°: 51.085820; E°: 4.954160
	BT237	Sint Laureins	N°: 51.27365; E°: 3.55620
	BT395	Kessel	N°: 51.153784; E°: 4.607329
	BT396	Christnach*	
	BT397	Doornik	N°: 50.607300; E°: 3.347340
C. nubeculosus	BT242	Knokke	N°: 51.340250; E°: 3.352130
	BT243	Betekom	N°: 51.00490; E°: 4.79240
	BT403	Varendonk	N°: 51.085820; E°: 4.954160
	BT404	Knokke	N°: 51.340250; E°: 3.352130
C. obsoletus	BT213	Betekom	N°: 51.00490; E°: 4.79240
	BT214	Betekom	N°: 51.00490; E°: 4.79240
	BT215	Sint Laureins	N°: 51.27365; E°: 3.55620
	ITM_OB	Belgium	Not defined
C. pulicaris	BT228	Knokke	N°: 51.340250; E°: 3.352130
	BT229	Zonhoven	N°: 50.976890; E°: 5.371410
	BT230	Kessel	N°: 51.153784; E°: 4.607329
	BT386	Oelegem	
	BT387	Manage	N°: 50.485950; E°: 4.215720
	BT388	Varendonk	N°: 51.085820; E°: 4.954160
C. punctatus	BT231	Betekom	N°: 51.00490; E°: 4.79240
	BT389	Knokke	N°: 51.340250; E°: 3.352130
	BT233	Kessel	N°: 51.153784; E°: 4.607329
	BT390	Varendonk	N°: 51.085820; E°: 4.954160
C. puncticollis	BT245	Betekom	N°: 51.00490; E°: 4.79240
C. riethi	BT246	Sint Laureins	N°: 51.27365; E°: 3.55620
C. scoticus	BT216	Kessel	N°: 51.153784; E°: 4.607329

	BT217	Betekom	N°: 51.00490; E°: 4.79240
	BT218	Betekom	N°: 51.00490; E°: 4.79240
	BT377	Oelegem	N°: 51.22662; E°: 4.61328
	BT378	Varendonk	N°: 51.085820; E°: 4.954160
	BT379	Betekom	N°: 51.00490; E°: 4.79240
	ITM_SC	Belgium	Not defined

Appendix C. GenBank accession numbers of the four sequences of the Obsoletus group species in this study.

Species	GenBank accession number
C. chiopterus	JF279448
C. dewulfi	JF279447
C. obsoletus	JF279444
C. scoticus	JF279445 and JF279446

Appendix D. GenBank accession numbers of the ITS1 sequences of the Culicidae, Psychodidae and Simuliidae species used for the local BLASTn search.

Species	**GenBank accession number**	**Country**
Culicidae		
Culex annulirostris	AY035882	Australia
Culex annulus	AF453488	China
Culex chidesteri	GU477612	Guatemala
Culex coronator	GU562347	USA
Culex erythrothorax	CEU33022	USA
Culex interrogator	GU562345	Guatemala
Culex nigripalpus	GU562871	USA
Culex palpalis	AY035883	Australia
Culex pipiens	EU306662	India
Culex pipiens quinquefasciatus	DQ341112	South Africa
Culex quinquefasciatus	GU562872	USA
Culex salinarius	CPU22144	USA
Culex sitiens	AY035885	Australia

Culex territans	CTU33036	USA
Culex thriambus	GU562874	USA
Culex tigripes	CTU33034	USA
Culex torrentium	CTU33039	USA
Culex tritaeniorhynchus	EF545246	China
Culex vishnui	AF165900	Japan
Psychodidae		
Lutzomyia ayacuchensis	AB479840	Japan
Lutzomyia barretoi	AB479872	Japan
Lutzomyia dysponeta	AB479873	Japan
Lutzomyia migonei	AB479866	Japan
Lutzomyia nevesi	AB479868	Japan
Lutzomyia panamensis	AB479865	Japan
Lutzomyia reburra	AB479870	Japan
Lutzomyia sallesi	AB479867	Japan
Lutzomyia serrana	AB479869	Japan
Lutzomyia shannoni	AB479871	Japan

Lutzomyia tortura	AB479844	Japan
Lutzomyia trapidoi	AB479852	Japan
Simuliidae		
Simulium aureohirtum	FJ538884	China
Simulium laciniatum	EU779824.1	Fiji
Simulium limbatum	EF535155	Brazil
Simulium oyapockense	AF427959	Brazil
Simulium roraimense	AF427961	Brazil
Simulium rufibasis	FJ538872	China